

SHORT COMMUNICATION

Environmental estrogen exposure disrupts sensory processing and nociceptive plasticity in the cephalopod *Euprymna scolopes*

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ABSTRACT

Endogenous estrogens affect multiple sensory systems, including those involved in processing noxious and painful stimuli. Extensive evidence demonstrates that estrogenic environmental pollutants have profound, negative effects on growth and reproductive physiology, but there is limited information about how estrogenic pollutants might affect sensory systems known to be modulated by endogenous estrogens. Here, we show that ethinyl estradiol, the most common artificial estrogen found in coastal marine environments, disrupts normal behavioral and neural responses to tissue injury in the sepiolid *Euprymna scolopes* (Hawaiian bobtail squid), which inhabits shallow tropical waters close to dense human habitation. Behavioral hypersensitivity and neural plasticity that occur normally after tissue injury were impaired both under chronic estrogen exposure beginning during embryogenesis and after a single, high dose co-incident with injury. This suggests that these naturally selected responses to injury, which function to protect animals from predation and infection risk, may be impaired by anthropogenic pollution.

KEY WORDS: Behavior, Ethinyl estradiol, EE2, Injury, Mechanosensation, Squid

INTRODUCTION

Estrogens are steroid hormones that affect nearly every physiological system in the body, including the peripheral and central nervous systems (McEwen et al., 2017). There is extensive evidence that endogenous and exogenous estrogens modulate responses to noxious sensory input (including pain experience) in mammals, where effects vary depending on multiple factors including the type of noxious sensation and the sex and life stage of the subject (Craft, 2007). Estrogen receptors (ERs) and physiological responses to estrogens are widespread throughout the animal kingdom, having been identified in numerous vertebrates and invertebrates, including cephalopods (D'Aniello et al., 1996; De Lisa et al., 2012; Keay et al., 2006). In octopuses, the ER has been localized to central nervous system lobes involved in learning, mechanosensory processing and nociceptive plasticity (De Lisa et al., 2012; Di Cristo et al., 2010), suggesting that any disruption to estrogen signaling may influence nociceptive processing in cephalopods, similar to effects known in mammals. Cephalopods express many of the same behavioral and neural responses to tissue injury and noxious sensory input as vertebrates, including

long-lasting nociceptive sensitization (Alupay et al., 2014; Crook et al., 2013; Howard et al., 2019), and behavioral hypervigilance (Crook et al., 2011), which is adaptive (Crook et al., 2014).

Exogenous estrogen exposure affects a range of complex behaviors in vertebrates, including play and social interactions (Zaccaroni et al., 2018), predator–prey interactions and aggression (Dang et al., 2017; Saaristo et al., 2010), and anxiety (Volkova et al., 2015; Zaccaroni et al., 2018). In fish, water-borne estrogenic pollutants have wide-ranging behavioral and physiological effects. Effects include alterations to behaviors that are also affected by nociceptive plasticity, such as aggression, anxiety and responses to predators (Dang et al., 2017; Dzieweczynski et al., 2014; Rearick et al., 2018; Volkova et al., 2015); however, whether similarly complex effects occur in aquatic invertebrates is less clear.

Synthetic estrogenic pollutants are increasingly present in ocean and freshwater systems throughout the world (Adeel et al., 2017; Aris et al., 2014; Bhandari et al., 2015), resulting from the growing discharge of sewage and industrial effluent from which estrogenic compounds are incompletely removed. Ethinyl estradiol (EE2) is the most common and potent of these substances, and numerous studies have now shown that even in very low concentrations, EE2 is damaging to the development and reproduction of aquatic organisms exposed to it (Bhandari et al., 2015; Jobling et al., 2004). Whether it can affect nociceptive processing in ways similar to endogenous estrogens or otherwise is not known.

Considering the well-known role of estrogens in modulating adaptive, injury-induced neural plasticity and behavior in mammals, the similarity of these responses across vertebrates and invertebrates, and the increasingly widespread occurrence of estrogenic pollution in coastal marine habitats, it is plausible that environmental estrogens can affect injury-induced neural plasticity and behavior in a wide range of coastal marine species. These effects are likely to extend to invertebrates such as cephalopods, which are important components of inshore food webs as eggs laid in the nearshore benthos, and juvenile and adult stages inhabiting shallow coastal waters. Injured and compromised prey represent preferred targets for many predatory species (Bowerman et al., 2010; Crook et al., 2014; Krumm et al., 2010; Semlitsch, 1990), and there is evidence that predation rates are affected by estrogen exposure in some fish species (Rearick et al., 2018); thus, any disruption to behavioral and neural plasticity processes that underlie adaptive responses to injury may have wide-ranging effects on marine ecosystems.

In this comparative study, we addressed these questions by exposing the coastal cephalopod *Euprymna scolopes* (Hawaiian bobtail squid) to two ecologically relevant doses of EE2 in early life. One dosing regimen maintained a continuous, low level (1 ng l^{-1}) of EE2 throughout development and into the hatchling and juvenile periods; the other exposed 2-week-old hatchlings to a single higher pulse of EE2 (20 ng l^{-1}) that then dissipated over time. These two regimens mimic levels of EE2 found commonly in

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coastal marine waters: the former within the range typically detected fairly constantly in inshore environments close to human habitation, the latter reflecting levels that might be experienced following a higher volume sewage effluent event (Bhandari et al., 2015). By subjecting half the animals in each treatment group to minor tissue injury at 2 weeks of age, we assessed behavioral and neurophysiological changes brought on by EE2 exposure, injury and the combination of the two, through the hatchling and juvenile period.

MATERIALS AND METHODS

Animals

Hawaiian bobtail squid (*Euprymna scolopes* Berry 1913) were captive bred F3 and F4 offspring originating from wild-caught *E. scolopes* collected in the waters of Maunaloa Bay, Kauai, Hawaii. Squid hatchlings ($N=155$) were fed *ad libitum* on live mysid shrimp (*Amerimysis bahia*). Squid are invertebrates and as such are excluded from regulatory oversight in the USA; thus, no IACUC protocol is associated with this study. However, we adhered to Directive 2010/63/EU and the ARRIVE guidelines for defining standards of care, humane endpoints and experimental procedures.

Rearing: tank set-up and exposure protocol

Eggs laid by captive-bred adults were collected from colony tanks daily. Within 24 h of being laid, clutches of *E. scolopes* eggs (~100–300 eggs per clutch) were split roughly in half by gentle separation of the egg mass using sterile surgical tools, then transferred to opaque tubs suspended in 20 gallon (76 l) aquarium tanks containing artificial seawater (salinity: 1.024 specific gravity; temperature: 24°C), which was aerated, filtered and pumped through the tubs at a rate of ~100 ml min⁻¹ prior to hatching, and 30 ml min⁻¹ after hatching. Salinity and pH were adjusted by continuous drip infusion of ultra-pure water (filtered via MilliQ) and two-part buffer (C-Balance Calcium Buffer, Two Little Fishes, Miami, FL, USA), as needed.

Each half-clutch of eggs was allocated to a different treatment to minimize effects of variation among clutches of different sizes or from different parents. Each treatment group included eggs from between six and nine clutches, all of which were laid by different females. Egg clutches hatched at 21 days post-laying. Females of *E. scolopes* mate with multiple males and store sperm, and clutches generally contain multiple paternities. Survival of *E. scolopes* hatchlings in the 10 day period from hatching to benthic settlement is highly variable (Hanlon et al., 1997), and is likely determined by

maternal age and condition, along with other unknown factors. In our experiments, survival to settlement percentages varied from ~20% to 60%, which is typical for this species. Although survival rates were variable among the treatment groups, there was no consistent pattern of survival indicating that this was associated with EE2 exposure.

Tanks designated for low, continuous exposure were dosed with 1 ng l⁻¹ EE2 (Sigma-Aldridge E4876-1G), diluted in 1 ml of vehicle (ethanol). Control tanks were dosed with the same volume of vehicle only. To counter the degradation of EE2 that occurs naturally in seawater, we replenished EE2 every 5 days throughout the experiment based on calculations from previous studies (Dzieweczynski et al., 2014; Robinson and Hellou, 2009).

Animals designated for the single, high-dose EE2 exposure were transferred from control tanks to separate tanks at 2 weeks of age. These tanks were then treated once only with a 20 ng l⁻¹ dose of EE2 in 1 ml vehicle, 24 h prior to tissue injury. All squid were group housed in their treatment tanks, as the small enclosures needed for single housing are associated with poorer survival. Thus, it was not possible to track individuals across the experiment, and all data are presented as pooled group means.

Injury procedure

We have shown previously that minor, contusive tissue injury in squid produces long-term nociceptive sensitization and behavioral changes that have positive fitness effects (Crook et al., 2011; Crook et al., 2014; Howard et al., 2019). We used the same model in this study. Half the squid in each EE2-exposure group received a minor tissue injury in the form of fin crush at 2 weeks post-hatching, while the other half were subjected to handling ('sham') only. Individual squid were removed from their home tank in a small beaker, placed in a 9 cm diameter glass Petri dish and then assigned randomly to the injured or uninjured group by card draw. For squid in the injury groups, the left fin was pinched with sterile, grooved forceps perpendicular to its edge, and held for 1–2 s. Injuries typically produced visible bruising of the tissue and some tearing of the tissue along the crush margin (see Fig. 1B for example). Typical behavioral responses were a characteristic defensive arm posture, which was sometimes accompanied by escape jetting or inking. Uninjured animals underwent the same procedure except that the forceps were only lightly touched against the fin. Animals were then returned to their home tanks. Injury extent was designed to be smaller than injuries commonly occurring in the wild, and all injuries healed within 1 week of being applied. There were no

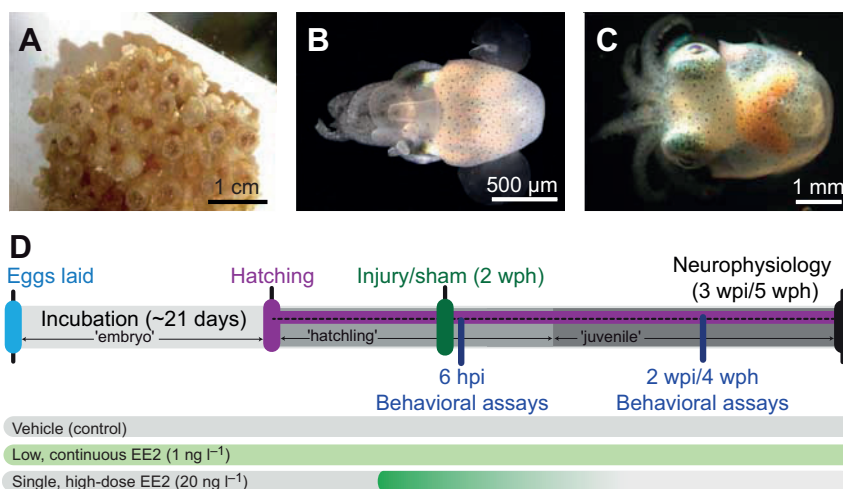


Fig. 1. Experimental subjects, treatments and timeline. (A) An unexposed egg clutch of *Euprymna scolopes* at 18 days incubation. (B) Hatchling *E. scolopes* in ventral view at 14 days post-hatching, with injury visible on the left fin. (C) Uninjured juvenile at 5 weeks of age. (D) The time line of experimental procedures and EE2 dosing. wph, weeks post-hatching; hpi/wpi, hours post-injury/weeks post-injury; EE2, ethinyl estradiol.

differences in survival, feeding or growth among the injured and uninjured groups.

Experiment 1: sensory threshold testing

Behavioral changes associated with injury in cephalopods typically include tactile hypersensitivity and escalated defensive behaviors (Alupay et al., 2014; Crook et al., 2011; Oshima et al., 2016). We tested for similar effects in this study, using tactile and vibratory sensory threshold tests at acute (6 h) and chronic (14 days) post-injury time points, when the animals were 2 weeks post-hatching (wph) and 4 wph, respectively. Animals were moved individually into a 9 cm diameter, glass Petri dish with a water depth of 1 cm, and allowed to acclimate for 1 min. All tests were videotaped from directly overhead, and the dish was placed in a visually isolated area on a vibration-isolation mat. Mechanosensory threshold testing was conducted using five von Frey filaments (Stoelting Co.) in ascending series (0.008, 0.02, 0.04, 0.07 and 0.16 g). Filaments were applied to the body of the squid beginning with the lightest filament (0.008 g) and continuing in ascending order until an avoidance behavior was observed, which was typically either escape jetting and/or inking.

Experiment 2: behavioral response to vibrational stimulus

One minute after the completion of mechanosensory threshold testing, the animals' response to a startling vibratory stimulus was tested. A 4 g weight was dropped from a fixed height of 45 cm above and 12 cm from the edge of the Petri dish, and the animal's response to the weight landing on the testing surface was recorded. The dish and weight drop-zone were atop a thick (3 cm) Styrofoam pad, which served to minimize vibrations from external sources during testing. Responses to the vibrational stimulus were somewhat different from the most frequent responses to the mechanical stimulus, and generally included color change and fin movement, rather than jetting or inking. The short interval between the two tests facilitated rapid screening of large numbers of squid, but may have led to responses to the vibrational test being affected by the prior mechanical stimulus. All behavioral tests were video-recorded for later analysis (see 'Data analysis and statistical procedures', below).

Experiment 3: peripheral nerve excitability

Measurements of peripheral nerve excitability were conducted 1 week after behavioral testing was complete, when hatchlings were 5 weeks of age. The 1 week interval was necessary to allow all behavioral tests for multiple cohorts to be completed, but we have shown previously that long-term nociceptive plasticity in this species is quite stable over time (Howard et al., 2019). Squid were killed by immersion in 330 mmol l⁻¹ MgCl₂ in filtered freshwater (Butler-Struben et al., 2018), then 5 min after respiration ceased, squid were decapitated and decerebrated, and mantles were dissected down the ventral midline. Viscera were removed by microdissection and the left and right stellate ganglia and pallial nerves were exposed and cleaned of overlying connective tissues. The mantle was bisected along the dorsal midline and readings from the left (injured/sham) and right (unmanipulated) side were taken separately. The tissue was washed in filtered, artificial seawater to remove residual magnesium chloride, and then the pallial nerve stump was drawn into a suction electrode for recording of evoked and spontaneous activity.

Signals were amplified on an A-M Systems Model 1700 extracellular amplifier, sampled at 10 kHz and digitized using an ADInstruments Powerlab 4/35 with LabChart Pro software. Evoked nerve activity was recorded in response to application of a von Frey

filament delivering force sufficient to activate nociceptors in this species of squid (0.02 g; see Howard et al., 2019). The filament was applied to three different locations on the mantle and held for 1 s after bending. After these baseline recordings, mantle tissue was crushed using grooved forceps ('*ex vivo* injury'). Tests of evoked nerve activity were repeated at 10 min after the *ex vivo* crush occurred.

Data analysis and statistical procedures

From recorded video footage we counted behavioral responses to the mechanical and vibrational stimuli. First, we screened all video recordings to identify a set of discrete behaviors that occurred in response to each stimulus in at least 10% of trials (thus, uncommon responses such as defensive arm postures were excluded from further analysis), then we quantified the frequency of these typical behaviors for each treatment group. The typical response to filament touch was jetting away or inking. The typical response to vibration was brief color change or movement of the fins and body. Data are presented as the proportion of squid per treatment group that produced these characteristic behavioral responses. Responses were counted independently by two trained observers not involved in the experiments and blind to the hypothesis being tested. Inter-rater reliability between the observers was 83%, meaning that when reviewing the same video, they scored behavioral responses identically 83% of the time. Where disagreements in rating were found, the primary experimenter (S.N.B.) and senior author (R.J.C.) re-scored responses. Statistical analyses were conducted in Prism 8.0 (GraphPad). We used Fisher's exact tests to compare proportions of animals in each group that responded to the mechanosensory and vibratory stimuli. The critical alpha was set at 0.05, and all *P*-values reported are two-tailed and adjusted for multiple comparisons using the Holm–Bonferroni method (Holm, 1979).

For electrophysiological data, the number of spikes produced above the noise threshold was counted for 1 s during the maximal firing period for evoked activity. Counts for the three replicate touches for each filament were averaged. Because we have previously shown that effects of injury are identical on injured and contralateral sides in two species of squid (Crook et al., 2013; Howard et al., 2019), right and left side values for a single animal were averaged to provide a technical replicate for each animal and control for any variation in tissue health between the two preparations. Count data deviated from expectations of normal distribution and were analyzed with Kruskal–Wallis tests with *post hoc* Mann–Whitney *U*-tests, or Friedman tests followed by Wilcoxon matched pairs *post hoc* tests, conducted in GraphPad Prism 8.0. The critical alpha was set at 0.05. All *P*-values reported are two-tailed and adjusted for multiple comparisons using the Holm–Bonferroni method.

RESULTS AND DISCUSSION

EE2 administered in either low, continuous (1 ng l⁻¹) or single, high (20 ng l⁻¹) doses induced complex, sometimes conflicting changes that altered age-appropriate (Seehafer et al., 2018) and injury-induced (Howard et al., 2019) behaviors. EE2 exposure also impaired the neural plasticity that drives a suite of heightened anti-predator behaviors that compensate for elevated predation risk in the post-injury period (Crook et al., 2014). The variability in behavioral and neural effects we report here may have been affected to some degree by the variation in sample size among the groups, which was caused by unequal survival through the settlement and juvenile period. Differences in hatchling survival were likely due primarily to maternal condition and egg provisioning (Hanlon et al., 1997) and were not associated in any consistent way with EE2 exposure.

Exposure to EE2 impairs defensive behavior in hatchlings

Continuous low doses of EE2 in seawater beginning in embryological development blunted responses of uninjured squid hatchlings to noxious mechanical stimulation, but this effect was not apparent in the single high-dose group. In comparison to unexposed/uninjured animals within each time point, only the low, continuous EE2/uninjured group showed a lower proportion of responses to touch in experiment 1 (Fisher’s exact test, $P=0.025$; Fig. 2A), 6 h after either injury or sham treatment. There was also an effect of continuous, low-dose EE2 exposure on the developmental shift in behavioral response previously reported in unmanipulated *E. scolopes* juveniles (Seehafer et al., 2018). Response to touch declined over time in all groups except those exposed to a low, continuous dose of estrogen (Fisher’s exact tests: unexposed/uninjured, $P=0.008$; unexposed/injured, $P=0.042$; single high-dose

EE2/uninjured, $P=0.019$; single high-dose/injured, $P=0.025$; low continuous EE2/uninjured, $P>0.05$; low continuous EE2/injured, $P>0.05$; sample sizes shown in Fig. 2A).

In contrast to defensive responses to touch, which declined over the course of post-embryonic development in unexposed squid, the typical course of responses to vibrational stimuli in the unexposed groups was an increasing trend over development. The proportion of unexposed animals that responded to the vibrational stimulus (experiment 2) increased significantly between testing at 6 h post-injury/sham and at 2 weeks post-injury/sham, whether uninjured or injured (Fig. 2B; Fisher’s exact tests: unexposed/uninjured, $P=0.03$; unexposed/injured, $P=0.003$). In contrast, in the two EE2-exposed groups, there appeared to be hypersensitivity to vibration at the hatchling stage. Behavioral responses to startling vibrational stimuli were similar in the two EE2-exposed groups, where uninjured

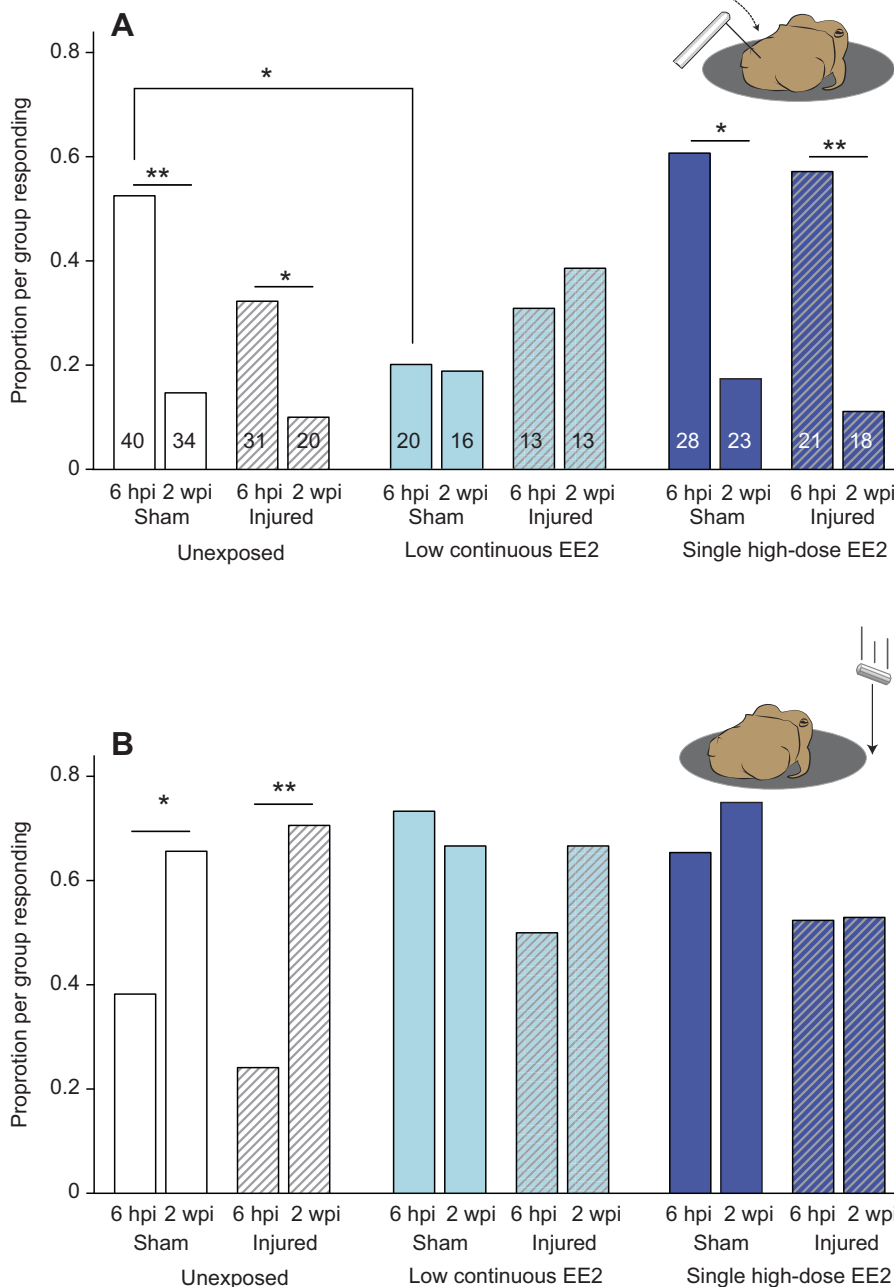


Fig. 2. Exposure to environmental estrogens impairs defensive behavior. (A) The proportion of squid in each treatment group responding to touch on the mantle with a von Frey filament (experiment 1) declined from hatchling (6 hpi/sham) to juvenile (2 wpi/sham) age classes, except in the groups chronically exposed to EE2. (B) The proportion of squid in each treatment group that respond to a startling vibrational stimulus (experiment 2) increased from the hatchling to juvenile age class in unexposed but not EE2-exposed groups. Similar to results from the experiment 1, there was no behavioral effect of injury evident. Numbers inside bars show sample size per group. Statistical analysis was performed using Fisher’s exact test with Holm–Bonferroni correction; * $P<0.05$, ** $P<0.01$.

hatchlings at 6 h post-injury/sham showed responses indistinguishable from behaviors observed in juveniles 2 weeks later. The vibration-response test was always conducted after the touch test, which may have resulted in responses to vibration being contingent on responses made to the mechanical stimulus. Previous studies have demonstrated that ink-borne alarm cues can influence subsequent squid behavior (Wood et al., 2008), and although inking was relatively uncommon in our study, we tested whether any positive response in the mechanosensory test was more likely to be associated with a positive response to the vibration test than a non-response. We found no significant association (Fisher's exact test, $P=0.84$), suggesting that the order of test presentation did not affect the results of the vibration test.

Thus, hatchling *E. scolopes* showed hypersensitivity to vibration under both EE2 exposures, but decreased response to touch only under the continuous low dose. In both behavioral assays, effects were most pronounced in hatchlings, with juveniles showing behavior more consistent with those of unexposed squid. Defensive behaviors in the hatchling stage are vitally important for cephalopods, which are under strong predation pressure both before and after hatching (Martins et al., 2018). In *E. scolopes*, the initial first few weeks of hatchling life are characterized by high mortality due to poor hunting success, which likely compounds predation risk through increased time spent hunting (Hanlon et al., 1997). It is possible, therefore, that EE2 negatively affects fitness of squid hatchlings through disruption of age-appropriate defensive

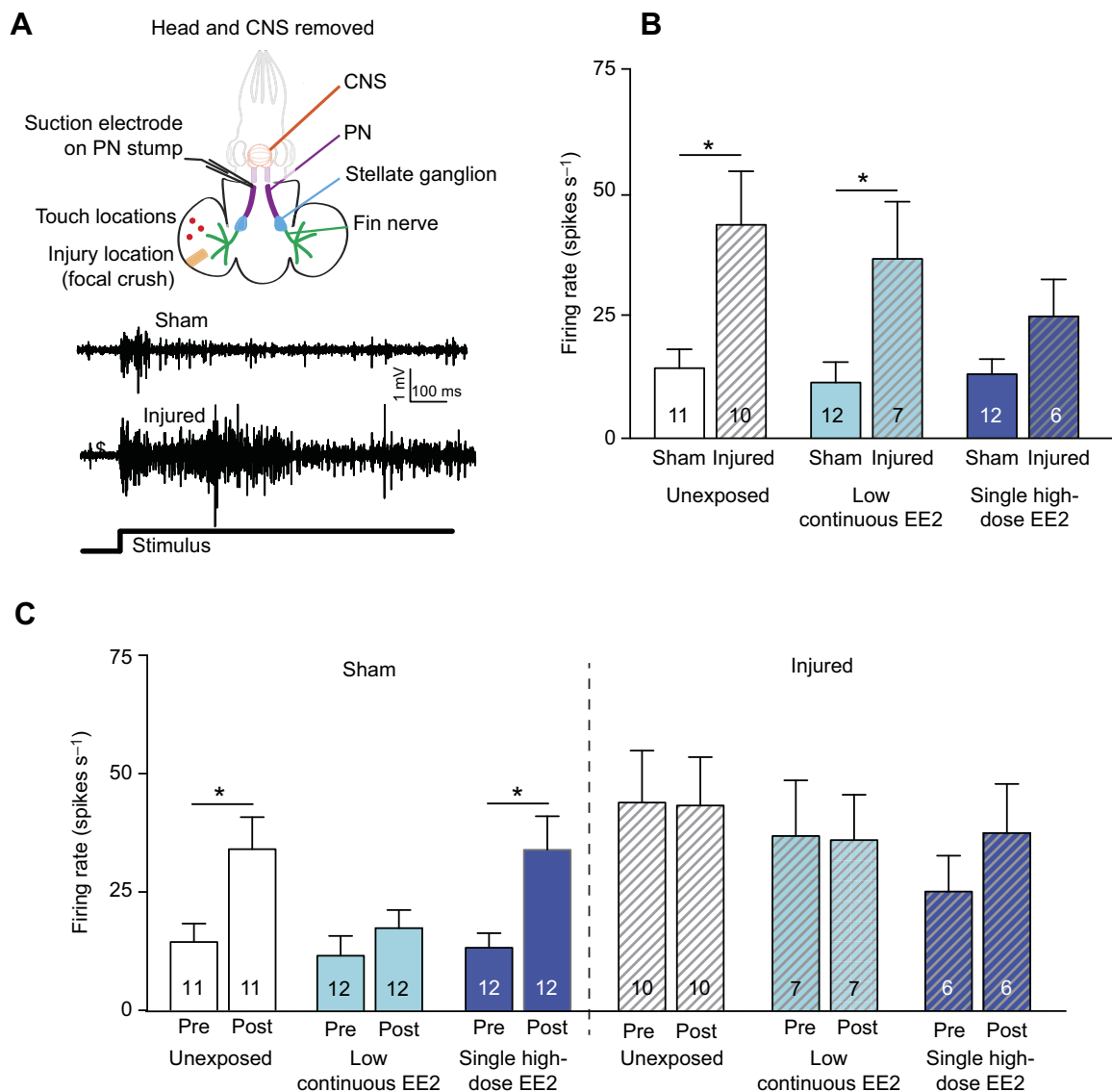


Fig. 3. Exposure to environmental estrogen impairs nociceptive plasticity. (A) Cartoon showing the basic anatomy of the peripheral nervous system of *E. scolopes* and the location of injury (orange), stimulation sites (red) and suction electrode placement (experiment 3). CNS, central brain; PN, pallial nerve. Example traces from a 1 s application of a von Frey filament are shown for sham and injured squid. (B) Injured squid from the unexposed and low, continuous dose group produced significantly more action potentials in response to mechanical stimulation compared with their uninjured counterparts, but animals that had been exposed to high-dose EE2 at the time of injury did not. Statistical analysis was performed using the Kruskal–Wallis test followed by *post hoc* Mann–Whitney *U*-tests with Holm–Bonferroni correction; $*P<0.05$. (C) Comparison of firing rates before (pre) and after (post) *ex vivo* tissue crush show that short-term sensitization is impaired by chronic estrogen exposure and prior injury. Statistical analysis was performed using the Friedman test followed by *post hoc* Wilcoxon matched-pairs test with Holm–Bonferroni correction; $*P<0.05$. Bars in B and C show means and errors bars are ± 1 s.e.m. Numbers inside bars are group sample sizes.

behaviors. Vibrational stimuli are used by near-hatching larvae in a number of species to predict predator presence, which can induce premature hatching (Warkentin, 2005). There are anecdotal reports of egg disturbance leading to premature hatching in cephalopods. In our culture facility, eggs close to hatching are kept isolated from disturbance; however, if estrogen exposure produces a heightened response to vibration before as well as after the hatching period (Komak et al., 2005), there is potential for premature hatching in the presence of estrogenic pollutants in the wild where egg disturbance is likely. Premature emergence of juvenile cephalopods is associated with dramatically reduced survival, which may have considerable impacts on wild population dynamics (Marthy et al., 1976).

High-dose EE2 coincident with injury impairs nociceptive plasticity

Recordings of pallial nerve activity (which carries nociceptive sensory information to the central brain; see Fig. 3A) in experiment 3 showed that a single, high dose of EE2 that coincided with tissue injury impaired development of long-term nociceptive sensitization. In both the unexposed group and the group exposed to a low, continuous dose of EE2, pallial nerves from animals that had received *in vivo* injury at 2 weeks post-hatching showed significantly higher firing rates than the respective sham group in response to application of a stiff von Frey filament (unexposed; Mann–Whitney *U*-test, $P=0.03$; low continuous dose; Mann–Whitney *U*-test, $P=0.03$; Fig. 3B), but there was no difference in the group exposed to high-dose EE2.

In contrast, after *ex vivo* tissue crush, which normally results in strong short-term sensitization (Crook et al., 2013; Howard et al., 2019), there were more spikes produced in the unexposed/sham and high dose/sham groups (unexposed/sham, Wilcoxon test, $P=0.037$; single high dose/sham, Wilcoxon test, $P=0.019$; Fig. 3C), but not in the low dose/sham group. Among the injured groups, there were no further increases in firing after *ex vivo* tissue crush (Fig. 3C), a pattern which has been shown previously for this species (Howard et al., 2019).

Long-term nociceptive sensitization is a normally occurring consequence of being injured in early life. Early life injury in *E. scolopes* and in a range of other species causes permanent changes to both the nervous system and defensive behavior (Howard et al., 2019; Schwaller and Fitzgerald, 2014) that are likely adaptive. Any disruption of long-term nociceptive plasticity is therefore likely to have negative consequences for survival of animals subject to predation pressure at any life stage. The absence of long-term nociceptive sensitization in the high-dose group at 5 weeks of age suggests that EE2 may suppress nociceptor activity at the time of injury, as activity-dependent mechanisms control the degree and duration of nociceptive sensitization in cephalopods (Alupay et al., 2014; Crook et al., 2013). The anti-nociceptive effects of transient, high concentrations of estrogens have been observed experimentally in rodents (Gintzler, 1980) and humans (Smith et al., 2006). Although high-dose EE2 appeared to interrupt the development of long-term sensitization, it did not appear to prevent *de novo* peripheral sensitization in the isolated fin-nerve preparation 3 weeks later.

Conclusions

Here, we show that environmental estrogen exposure has various detrimental effects on mechanosensation, age-appropriate defensive behaviors and responses to tissue injury in squid. Although the effects are likely more complex in the wild where multiple stressors act on squid at any given time, here in this controlled, captive environment we found potentially important effects on behaviors

and neural plasticity critical to fitness. Impairments to fitness-protecting defensive behaviors result in increased predation risk (Crook et al., 2014; Hwang et al., 2007; Perrot-Minnot et al., 2017). Deficits in sensory processing and blockade of neural plasticity after exposure to environmentally relevant levels of estrogenic pollution are likely to impair a range of behaviors that allow squid (and potentially many other similarly affected marine species) to make optimal choices to avoid predation and hunt effectively at different life stages. Estrogenic pollutants may therefore play important and currently underappreciated roles in the ongoing disruption to inshore marine ecosystems.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.N.B., R.J.C.; Methodology: S.N.B., R.J.C.; Formal analysis: S.N.B., R.J.C.; Investigation: S.N.B.; Resources: R.J.C.; Data curation: S.N.B., R.J.C.; Writing - original draft: S.N.B., R.J.C.; Writing - review & editing: S.N.B., R.J.C.; Visualization: S.N.B., R.J.C.; Supervision: R.J.C.; Project administration: R.J.C.; Funding acquisition: S.N.B., R.J.C.

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